USER GUIDE

Applause® WT-Amp Plus ST System

PART NO. 5510

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I. Introduction

A. Background

The Applause® WT-Amp Plus ST System provides a fast, simple and cost-effective method for preparing amplified cDNA for global gene expression analysis on Affymetrix® GeneChip® Gene 1.0 ST and Exon 1.0 ST Arrays. Powered by Ribo-SPIA® technology, a rapid, simple and sensitive RNA amplification process developed by NuGEN, the Applause WT-Amp Plus ST System enables the generation of microgram quantities of cDNA in approximately seven hours. Amplification is initiated at the 3′ end as well as randomly throughout the transcriptome, making the Applause WT-Amp Plus ST System an ideal choice for use with whole transcript array designs such as the Gene 1.0 ST and Exon 1.0 ST Array.

The Applause WT-Amp Plus ST System (Part No. 5510) provides optimized reagent formulations and a protocol to process total RNA samples.

B. How the Applause WT-Amp Plus ST System Works

The Applause WT-Amp Plus ST System utilizes Ribo-SPIA technology that produces amplified cDNA from total RNA (see Figure 1).

1. **Generation of First Strand cDNA (1 hour)**
   First strand cDNA is prepared from a minimum of 50 ng of high-quality total RNA sample using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase (RT). The primers have a DNA portion that hybridizes either to the 5′ portion of the poly(A) sequence or randomly across the transcript. RT extends the 3′ DNA end of each primer generating first strand cDNA. The resulting cDNA/mRNA hybrid molecule contains a unique RNA sequence at the 5′ end of the cDNA strand.

2. **Generation of a DNA/RNA Heteroduplex Double-stranded cDNA (1.5 hours)**
   Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second strand, which includes DNA complementary to the 5′ unique sequence from the first strand chimeric primers. The result is a double-stranded cDNA with a unique DNA/RNA heteroduplex at one end.

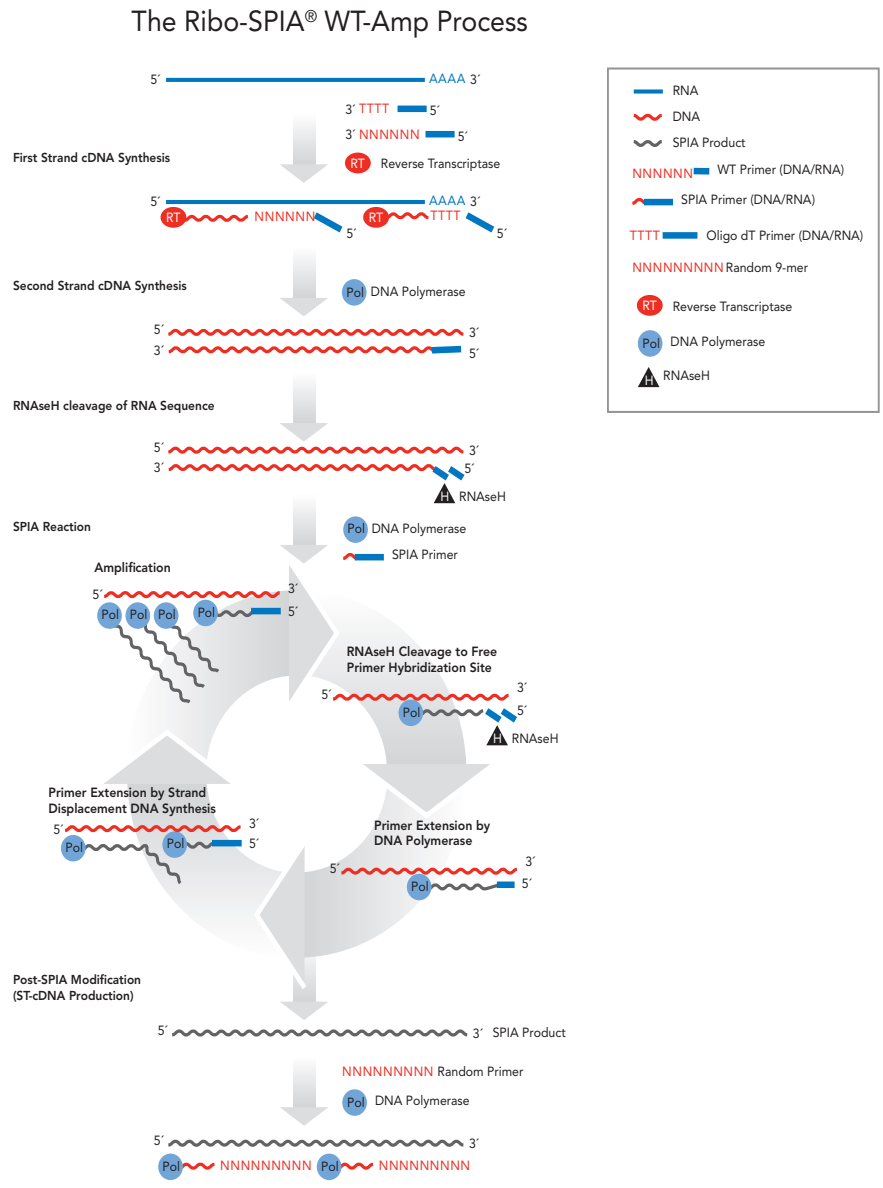
3. **SPIA® Amplification (1.5 hours)**
   SPIA is a robust isothermal strand displacement amplification process developed by NuGEN. The process uses a SPIA DNA/RNA chimeric primer, DNA polymerase and RNase H in a homogeneous isothermal assay that provides highly efficient amplification of DNA sequences. RNase H degrades RNA in the DNA/RNA heteroduplex at the 5′ end of the first cDNA strand. This exposes a DNA sequence that is available for binding to the SPIA DNA/RNA chimeric primer. DNA polymerase initiates replication at the 3′ end of the primer, displacing the existing forward strand. The RNA portion at the 5′ end of the newly synthesized strand is again removed by RNase H, exposing the unique priming site for initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated, resulting in rapid accumulation of cDNA with sequence complementary to the original mRNA.

I. Introduction

4. Post-SPIA Modification, Purification and QC (3 hours)

The Post-SPIA Modification process completes the amplification process. The first step allows the random primers to anneal to the single-stranded, antisense cDNA target. The second step utilizes DNA polymerase to extend the annealed primers, producing ST-cDNA targets appropriate for use with GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays.

Figure 1. The Ribo-SPIA Whole Transcriptome RNA Amplification Process



I. Introduction

C. Performance Specifications

The Applause WT-Amp Plus ST System synthesizes microgram quantities of ST-cDNA starting with a total RNA input of at least 50 ng. In approximately seven hours, the system produces sufficient cDNA for labeling and subsequent hybridization to GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays. The size of the majority of the cDNA products produced by the amplification process is between 0.1 and 2.0 kilobases.

D. Quality Control

Each Applause WT-Amp Plus ST System lot is tested to meet specifications of yield and array performance.

E. Storage and Stability

The Applause WT-Amp Plus ST System is shipped on dry ice and should be unpacked immediately upon receipt. All components should be stored at –20°C in a freezer without a defrost cycle.

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months. NuGEN has not yet established long-term storage stability for the Applause WT-Amp Plus ST System.

F. Material Safety Data Sheet (MSDS)

An MSDS for this product is available on the NuGEN website at http://www.nugeninc.com/nugen/index.cfm/support/user-guides/.

II. Kit Components

A. Reagents and Supplies Provided

Table 1. First Strand cDNA Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
First Strand Primer Mix	S01262	Blue	A1 VER 4
First Strand Buffer Mix	S01256	Blue	A2 VER 3
First Strand Enzyme Mix	S01040	Blue	A3 VER 1

Table 2. Second Strand cDNA Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
Second Strand Buffer Mix	S01257	Yellow	B1 VER 3
Second Strand Enzyme Mix	S01126	Yellow	B2 VER 2
Enhancement Enzyme Mix	S01119	Yellow	B3 VER 1

Table 3. SPIA Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
SPIA Primer Mix	S01263	Red	C1 VER 8
SPIA Buffer Mix	S01258	Red	C2 VER 8
SPIA Enzyme Mix	S01260	Red	C3 VER 5

Table 4. Post-SPIA Modification Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
Primer Mix	S01265	Violet	E1 VER 1
Buffer Mix	S01266	Violet	E2 VER 2
Enzyme Mix	S01383	Violet	E3 VER 1

Table 5. Additional Reagents

COMPONENT	PART NUMBER	VIAL CAP	VIAL NUMBER
Nuclease-free Water	S01001	Green	D1

Note: The reagents in the Applause WT-Amp Plus ST System are similar to reagents in NuGEN's other kits. However, unless the part numbers are identical, these reagents do not have exactly the same composition and, therefore, are not interchangeable. Do not exchange reagents between different kits, as it will adversely affect performance.

B. Additional Reagents, Supplies and Equipment

Required Materials

- Equipment
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge or centrifuge for individual 0.2 mL tubes, strip tubes and PCR plates
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, and 200–1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, heated lid, and 100 µL reaction capacity
  - Appropriate spectrophotometer and cuvettes, or a Nanodrop® UV-Vis Spectrophotometer
- Reagents
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps

II. Kit Components

- Supplies and Labware
  - Nuclease-free pipette tips
  - 1.5 mL and 0.5 mL RNase-free microcentrifuge tubes
  - 0.2 mL individual thin wall PCR tubes, 8 X 0.2 mL strip PCR tubes or 0.2 mL thin wall PCR plates
  - QIAGEN® MinElute® Reaction Cleanup Kit (Cat. #28204)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

Optional Materials

- Agilent® 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of RNA
- Real Time PCR system
- Cleaning solutions such as RNaseZap® (Ambion, Cat.#AM9780) and DNA-OFF™ (MP Biomedicals, Cat.#QD0500)

To Order:

- Ambion Inc., www.ambion.com
- MP Biomedicals, www.mpbio.com
- New England BioLabs, www.neb.com
- QIAGEN Inc., www.qiagen.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- USB Corporation, www.usbweb.com

III. Planning the Experiment

A. Input RNA Requirements

It is important to assess the quality of your RNA sample prior to planning your amplification. The Applause WT-Amp Plus ST System is designed to be used with high quality RNA samples.

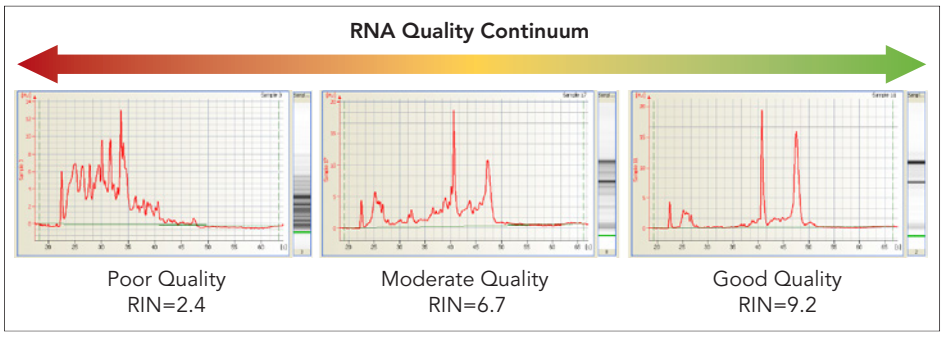
1. **RNA Quantity**
   The Applause WT-Amp Plus ST System is designed to use purified total RNA samples in the input range from 50 to 200 ng.

2. **RNA Purity**
   Purified total RNA samples must be free of contaminating proteins and other cellular material, organic solvents (including phenol and ethanol) and salts used in many RNA isolation methods. Use of a commercially available system for preparation of RNA that does not require organic solvents is recommended. If a method such as TRIzol® is used, we recommend employing an additional column purification step after isolation in order to remove any residual organics. One measure of RNA purity is the ratio of absorbance readings at 260 and 280 nm. The A260:A280 ratio for RNA samples of acceptable purity should be in excess of 1.8. RNA samples with lower ratios may result in poor amplification.

3. **RNA Integrity**
   Purified total RNA samples of high molecular weight with little or no evidence of degradation are required for use with this product.

   RNA integrity can be determined using the Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip® or RNA 6000 Pico LabChip. The RNA Integrity Number (RIN), available in the Bioanalyzer 2100 Expert Software, provides an index of RNA quality that can be helpful in triaging purified RNA samples of varying integrity prior to amplification.

Figure 2. This illustration of RNA quality variation shows Bioanalyzer traces of three different RNAs with varying degrees of quality.



4. **User Quality Control Guidelines for RNA samples**
   The inclusion of positive control RNA samples is an essential tool in evaluating the success of an amplification experiment. In the absence of successful positive con-

trol RNA amplification, it may be difficult or impossible to troubleshoot amplification issues.

5. **DNase Treatment**
   The use of DNase-treatment is highly recommended when using purified RNA samples. Contaminating genomic DNA will interfere with accurate quantitation of RNA samples and may negatively impact detection sensitivity and data quality. Refer to the Appendix for examples of DNase treatment protocols that have been used successfully.

6. **Carrier use for RNA isolation**
   We strongly recommend against the use of nucleic acid based carriers during RNA purification because many have been shown to produce cDNA product in first strand synthesis. We also advise against the use of glycogen in RNA isolation, as it inhibits reverse transcription. For the latest information regarding other carriers, contact our technical services team.

B. Using RNase-free Techniques

RNase contamination through reagents and work environment will lead to experimental failure. Follow these guidelines to minimize RNases in the workspace:

1. Wear disposable gloves and change them frequently.
2. Avoid touching surfaces or materials that could introduce RNases.
3. Use reagents provided. Substitutions may introduce RNases.
4. Clean work areas and instruments, including pipettes, with commercially available cleaning reagents, such as RNaseZap.
5. Use only new RNase-free pipette tips and microcentrifuge tubes.
6. Use a work area specifically designated for RNA work and do not use other high copy number materials in the same area.

C. RNA Storage

RNA samples should be stored at –80°C. Avoid frequent freeze/thaw cycles of RNA, as RNA degradation may result.

D. Amplified cDNA Storage

The amplified ST-cDNA produced by the Applause WT-Amp Plus ST System may be stored at –20°C.

IV. Amplification Protocols

A. Overview

The Ribo-SPIA amplification process used in the Applause WT-Amp Plus ST System is performed in five stages:

1. First strand cDNA synthesis	1 hour
2. Second strand cDNA synthesis and enhancement	1.5 hours
3. SPIA amplification	1.5 hours
4. Post-SPIA modification	2 hours
5. cDNA purification and quantitation	1 hour
Total time to prepare amplified cDNA	**~7 hours**

Applause components are color coded, with each reagent vial linked to a specific process stage. Performing each stage requires the simple addition of a master mix or other reagents, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

B. Protocol Notes

- It is important to set up no fewer than eight reactions at a time. This will ensure that you are not pipetting very small volumes (see the second strand synthesis section) below the effective range of air displacement pipetting technologies. For this reason, setting up fewer than eight reactions can lead to poor performance.
- Thaw components used in each step and immediately place them on ice as indicated in this user guide. It is best not to thaw reagents for all steps at once. The reagent color coding can be a guideline for appropriate reagent grouping.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, in rare instances a precipitate is observed. It is important that it be re-dissolved completely prior to use. You may gently warm the Buffer Mix for two minutes at room temperature followed by brief vortexing. Do not warm any enzyme or primer mixes.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

- When preparing master mixes, use the minimal amount of extra material to ensure there are sufficient reagents for 24 reactions. Typically an overage factor of 10% is acceptable. For example, if making a master mix for eight reactions use a factor of 8.8x when calculating the master mix volumes.
- Components and reagents from other Ovation® System, WT-Ovation® System or Applause System products should not be used with this product.
- **Caution:** The Enhancement Enzyme Mix, B3 (patent pending) contains a heat-labile RNase I enzyme. When using this reagent take care not to splash or contaminate gloves, bench or pipettes. Preferably use a dedicated pipette to measure out B3.
- Due to the high sensitivity inherent in this amplification system we strongly recommend taking measures to minimize the potential for the carryover of previously amplified SPIA cDNA into new amplification reactions. The two steps to accomplish this are: 1. Designating separate workspaces for "pre-amplification" and "post-amplification" steps and materials and 2. Implementing routine clean-up protocols for workspaces as standard operating procedure. A detailed set of these recommendations is given in the Appendix.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, and with a capacity of 100 µL reaction volume. Prepare the programs shown in Table 6, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100°C. For thermal cyclers with a fixed temperature heated lid (e.g., ABI GeneAmp® PCR 9600 and 9700 models) use the default settings (typically 100 to 105°C).

IV. Amplification Protocols

Table 6. Thermal Cycling Protocols

FIRST STRAND cDNA SYNTHESIS	
Program 1 Primer Annealing	65°C – 5 min, hold at 4°C
Program 2 First Strand Synthesis	4°C – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C
SECOND STRAND cDNA SYNTHESIS	
Program 3 Second Strand Synthesis	4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 70°C – 5 min, hold at 4°C
POST-SECOND STRAND ENHANCEMENT	
Program 4 Post-Second Strand Enhancement	4°C – 1 min, 37°C – 15 min, 80°C – 20 min, hold at 4°C
SPIA AMPLIFICATION	
Program 5 SPIA Amplification	4°C – 1 min, 47°C – 90 min, 95°C – 5 min, hold at 4°C
POST-SPIA MODIFICATION	
Program 6 Post-SPIA Modification I	4°C – 1 min, 37°C – 15 min, 95°C – 5 min, hold at 4°C
Program 7 Post-SPIA Modification II	4°C – 1 min, 30°C – 10 min, 42°C – 60 min, 75°C – 10 min, hold at 4°C

D. First Strand cDNA Synthesis

Important: Carry out Protocol D (First-Strand cDNA Synthesis) through Protocol G, step 7 (SPIA Amplification) in a pre-amplification workspace using dedicated pre-amplification consumables and equipment. Wipe all surfaces, equipment and instrumentation with a DNA removal solution such as DNA-OFF (MP Biomedicals, Cat. #Q0500) to avoid the potential introduction of previously amplified cDNA into new amplifications. For more information on our recommendations for workflow compartmentalization and routine lab cleanup please refer to Appendix E. If you have any questions on this important topic, please contact the NuGEN Technical Services Team: techserv@nugeninc.com or (888) 654-6544).

1. Obtain the First Strand Primer Mix (blue: A1), First Strand Buffer Mix (blue: A2), First Strand Enzyme Mix (blue: A3) and Nuclease-free Water (green: D1) from –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature. Mix by vortexing, spin and place on ice. Leave Nuclease-free Water at room temperature.
4. Add 5 µL of total RNA sample (50 to 200 ng) to a 0.2 mL PCR tube.
5. Add 2 µL of A1 to each reaction tube.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 6):

   65°C – 5 min, hold at 4°C
8. Remove the tubes from the thermal cycler and place on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining A2 and A3 in a 0.5 mL capped tube, according to the volumes in Table 7.

Table 7. First Strand Master Mix (volumes listed are for a single reaction)

FIRST STRAND BUFFER MIX (BLUE: A2 VER 3)	FIRST STRAND ENZYME MIX (BLUE: A3 VER 1)
2.5 µL	0.5 µL

10. Add 3 µL of the First Strand Master Mix to each tube.
11. Mix by pipetting 5 times, spin and place on ice.

12. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 6):

    4°C – 1 min, 25°C – 10 min, 42°C – 10 min, 70°C – 15 min, hold at 4°C

13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

E. Second Strand cDNA Synthesis

1. Obtain the Second Strand Buffer Mix (yellow: B1), Second Strand Enzyme Mix (yellow: B2) and Enhancement Enzyme Mix (yellow: B3) from –20°C storage.
2. Spin down the contents of B2 and B3 and place on ice.

! *Do not return B1 VER 3 buffer to the freezer, as it is required for the next step as well.*

3. Thaw reagent B1 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by combining B1 and B2 in a 0.5 mL capped tube, according to the volumes shown in Table 8.

Table 8. Second Strand Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 3)	SECOND STRAND ENZYME MIX (YELLOW: B2 VER 2)
9.75 µL	0.25 µL

5. Add 10 µL of the Second Strand Master Mix to each First Strand reaction tube.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 3 (Second Strand cDNA Synthesis; see Table 6):

    4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 70°C – 5 min, hold at 4°C

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
9. Continue immediately with the Post-Second Strand Enhancement proctocol.

F. Post-Second Strand Enhancement

1. Make a master mix by combining B1 and B3 in a 0.5 mL capped tube, according to the volumes shown in Table 9.

Table 9. Enhancement Master Mix (volumes listed are for a single reaction)

SECOND STRAND BUFFER MIX (YELLOW: B1 VER 3)	REACTION ENHANCEMENT MIX (YELLOW: B3 VER 1)
3.7 µL	0.3 µL

Note: In cases where the reactions will be stored at -20°C overnight prior to carrying out the Post-SPIA Modification protocol, make only half the required Enhancement Master Mix at this point (i.e., use only 1.85 µL B1 and 0.15 µL B3 per reaction). The other half of this master mix will need to be made fresh on day 2. We do not recommend storing this master mix overnight for use the next day.

2. Add 2 µL of the Enhancement Master Mix to each Second Strand reaction tube.
3. Mix by pipetting 5 times with a pipet set to 15 µL, spin and place on ice.

   Note: Save the remaining Enhancement Master Mix on ice. It will be used in the Post-SPIA Modification Protocol (Sections IV, H & K).
4. Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (Post-Second Strand Enhancement; see Table 6):

   4°C – 1 min, 37°C – 15 min, 80°C – 20 min, hold at 4°C
5. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue immediately with the SPIA Amplification protocol.

G. SPIA® Amplification

1. Obtain the SPIA Buffer Mix (red: C2), SPIA Primer Mix (red: C1) and SPIA Enzyme Mix (red: C3) from –20°C storage.
2. Thaw C3 on ice and mix the contents by inverting gently 5 times. Ensure the enzyme is well mixed without introducing bubbles, spin and place on ice.
3. Thaw reagents C1 and C2 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by sequentially combining C2, C1 and C3 in a 0.5 mL capped tube, according to the volumes shown in Table 10.

   Note: Ensure the addition of C3 is at the last moment, and that the master mix is mixed thoroughly before aliquoting.

Table 10. SPIA Master Mix (volumes listed are for a single reaction)

SPIA BUFFER MIX (RED:C2 VER 8)	SPIA PRIMER MIX (RED:C1 VER 8)	SPIA ENZYME MIX (RED:C3 VER 5)
2.5 µL	2.5 µL	5 µL

5. Add 10 µL of the SPIA Master Mix to the Post-Second Strand Enhancement reaction.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-cooled thermal cycler programmed to run Program 5 (SPIA Amplification, see Table 6):

   4°C – 1 min, 47°C – 90 min, 95°C – 5 min, hold at 4°C
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice. **Do not re-open the tubes in teh pre-amplification workspace.**

Important: At this point the tubes should be removed from the pre-amplification workspace. Carry out all remaining steps in a post-amplification workspace using dedicated post-amplification consumables and equipment. Take care to avoid the introduction of previously amplified cDNA into your pre-amplification workspace. For more information on our recommendations for workflow compartmentalization and routine lab cleanup, please refer to Appendix E of this User Guide. If you have any questions on this important topic, please contactthe NuGEN Technical Services Team: techserv@nugeninc.com or (888) 654-6544).

9. (Optional) If qPCR will be performed on the amplification products, remove an aliquot of the SPIA cDNA at this point.
10. Continue immediately with the Post-SPIA Modification I protocol or store the SPIA reactions at –20°C overnight prior to continuing.

H. Post-SPIA Modification I

1. Obtain the Primer Mix (Violet: E1), Buffer Mix (Violet: E2) and Enzyme Mix (Violet: E3) from –20°C storage.
2. Spin down the contents of E3 and place on ice.
3. Thaw E1 and E2 at room temperature, mix by vortexing, spin and place on ice.

4. Retrieve the remaining Post-Second Strand Enhancement Mix from step IV. F. that was set aside on ice.

   Note: In cases where the reactions have been stored at -20°C overnight prior to carrying out the Post-SPIA Modification Protocol, make the second half of the Enhancement Master Mix fresh at this point by mixing 1.85 µL B1 and 0.15 µL B3 per reaction. We do not recommend storing this Master Mix overnight for use the next day.

5. Make a master mix by combining the Enhancement Master Mix and E1 in a 0.5 mL capped tube, according to the volumes shown in Table 11.

Table 11. Post-SPIA Modification I Master Mix (volumes listed are for a single reaction)

ENHANCEMENT MASTER MIX	PRIMER MIX (VIOLET: E1 VER 1)
2 µL	6 µL

6. Add 8 µL of the Post-SPIA Modification I Master Mix to the SPIA Amplification reaction.
7. Mix by pipetting 5 times with a pipette set to 20 µL, spin and place on ice.
8. Place the tubes in a pre-cooled thermal cycler programmed to run Program 6 (Post-SPIA Modification I, see Table 6):

   4°C – 1 min, 37°C – 15 min, 95°C – 5 min, hold at 4°C

9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
10. Continue immediately with the Post-SPIA Modification II protocol.

I. Post-SPIA Modification II

The E3 Enzyme Mix is quite viscous. Please take care and pipette this mix slowly into the mix.

1. Make a master mix by combining E2 and E3 in a 0.5 mL capped tube, according to the volumes shown in Table 12.

Table 12. Post-SPIA Modification II Master Mix (volumes listed are for a single reaction)

BUFFER MIX (VIOLET: E2 ver 2)	ENZYME MIX (VIOLET: E3 VER 1)
6 µL	6 µL

2. Add 12 µL of the Post-SPIA Modification II Master Mix to the Post-SPIA Modification I reaction.
3. Mix by pipetting 5 times with a pipette set to 30 µL, spin and place on ice.
4. Place the tubes in a pre-cooled thermal cycler programmed to run Program 7 (Post-SPIA Modification II, see Table 6):

   4°C – 1 min, 30°C – 10 min, 42°C – 60 min, 75° – 10 min, hold at 4°C
5. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue to the Purification of ST-cDNA protocol, or store the cDNA at –20°C.

J. Purification of ST-cDNA

The ST-cDNA should be purified using QIAGEN's MinElute Reaction Cleanup Kit, Catalog #28204. Instructions are for a single reaction.

Important notes:

- The ERC buffer is considered hazardous, according to QIAGEN, and an MSDS may be consulted.
- Add the appropriate amount of 100% ethanol to Buffer PE before use (see bottle label for volume).
- All centrifuge steps are carried out at maximum speed in a conventional table-top microcentrifuge at room temperature.

1. Into a clean, labeled 1.5 mL microcentrifuge tube, add 300 µL of Buffer ERC from the QIAGEN kit.
2. Add the entire volume (52 µL) of the Post-SPIA Modification II reaction to the tube.
3. Vortex for 5 seconds, then spin briefly.
4. Obtain and label a MinElute spin column and place it into a collection tube.
5. Load the entire volume of sample/buffer mixture onto the column.
6. Centrifuge for 1 minute at maximum speed in a microcentrifuge.
7. Discard the flow-through and replace the column in the same collection tube.
8. Add 750 µL of Buffer PE the column.
9. Centrifuge for 1 minute at maximum speed.
10. Discard the flow-through and replace the column in the same collection tube.
11. Centrifuge for an additional 2 minutes at maximum speed to remove all residual Buffer PE.

! *100% ethanol must be added to the QIAGEN Buffer PE upon first use. Failure to do so will result in low amplification yields.*

Note: Residual ethanol from the wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Discard the flow-through with the collection tube. Blot the column onto clean, absorbant paper to remove any residual wash buffer from the tip of the column.

    Note: Blotting the column tip MUST be done prior to transferring the column to a clean tube. Failure to do so may result in a small quantity of wash buffer in your final eluted sample.

13. Place the column into a clean, labeled 1.5 mL microcentrifuge tube.

Use nuclease-free water at room temperature to elute sample

14. Add 15 µL of room temperature, Nuclease-free Water (green: D1) from the NuGEN kit to the center of each column.

    Note: Ensure that the water is dispensed directly onto the membrane for complete elution of bound cDNA.

15. Let the column stand for 1 minute at room temperature.
16. Centrifuge for 1 minute at maximum speed.
17. If two columns were used per sample, pool the eluates.
18. Discard the column and measure the volume recovered. There should be approximately 12 to 15 µL of purified SPIA cDNA.
19. Mix the sample by vortexing, then spin briefly.
20. Continue to the Measuring ST-cDNA Yield and Purity protocol or store purified ST-cDNA at –20°C.

K. Measuring ST-cDNA Yield and Purity

Note: You must purify the ST-cDNA before measuring yield and purity.

1. Mix the purified ST-cDNA sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance at 260, 280 and 320 nm of your ST-cDNA product. You may need to make a 1:20 dilution of the ST-cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the A320 value from both A260 and A280 values. The adjusted (A260 – A320) / (A280 – A320) ratio should be > 1.8.
4. Yield: Assume 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/mL.

   To calculate:

   (A260 – A320 of diluted sample) X (dilution factor) X 33 (concentration in µg/mL of a 1 absorbance unit solution) X 0.03 (final volume in mL) = total yield in micrograms

5. Alternatively, you may measure the concentration and purity of ST-cDNA with a Nanodrop, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/mL as the constant.
6. The purified ST-cDNA may be stored at –20°C.

V. Labeling with the Encore Biotin Module

A. Encore® Biotin Module Overview

The Encore Biotin Module (Part No. 4200) is used to label the ST-cDNA generated by the Applause WT-Amp Plus ST System in preparation for hybridization on Affymetrix GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays. Two Encore Biotin Module 12-reaction kits (Part No. 4200-12) are required to process the 24 cDNA targets from the Applause WT-Amp Plus ST Syatem. Follow the protocol given below when using the Applause WT-Amp Plus ST System with the Encore Biotin Module.

The cDNA labeling procedure is performed in two stages:

1. cDNA fragmentation	0.5 hours
2. Biotin labeling	1.25 hours
Total time to label amplified cDNA	**1.75 hours**

B. Protocol Notes

- Thaw only the components used in each step and immediately place them on ice.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve it completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing. Do not warm any enzyme mixes.
- FL3 labeling buffer may appear to have pink coloration, this is normal.
- The reagent volumes recovered greatly depend on the number of batches processed with each kit. Set up no fewer than three reactions at a time.
- When placing small amounts of reagents into reaction mix, gently pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume, at least half of total reaction mix volume. Repeat a minimum of five times to ensure complete mixing.
- Allow the thermal cycler to reach the initial incubation temperature before placing samples in the block.

C. Preparing cDNA Samples

Purified ST-cDNA from the Applause WT-Amp Plus ST System is ready for labeling with the Encore Biotin Module protocol. Use 4 to 5 µg of ST-cDNA as input into the Encore Biotin Module.

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes, equipped with a heated lid, with a capacity of 100 µL reaction volume. Prepare the 2 programs, shown

in Table 13, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100°C. For thermal cyclers with a fixed temperature heated lid (e.g., ABI GeneAmp PCR 9600 and 9700 models) use the default settings (typically 100 to 105°C).

Table 13. Thermal Cycler Programming

PROGRAMMING DETAILS	
Program 8 cDNA Fragmentation	37°C – 30 min, 95°C – 2 min, hold at 4°C
Program 9 Biotin Labeling	37°C – 60 min, 70°C – 10 min, hold at 4°C

E. cDNA Fragmentation

1. Obtain the Fragmentation Buffer Mix (Orange: FL1) and Fragmentation Enzyme Mix (Orange: FL2) from –20°C storage.
2. Spin down the contents of FL2 and FL5 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice.
4. Add 25 µL of the purified ST-cDNA (4 to 5 µg) to a 0.2 mL PCR tube.
5. Make a master mix by combining FL1 and FL2 in a 0.5 mL capped tube, according to the volumes shown in Table 14.

! *Use Fragmentation Master Mix immediately after preparation.*

! *Mix by pipetting and spin down the master mix briefly. Place on ice. Use master mix immediately.*

Table 14. Fragmentation Master Mix (volumes listed are for a single reaction)

FRAGMENTATION BUFFER MIX (ORANGE: FL1)	FRAGMENTATION ENZYME MIX (ORANGE: FL2)
5 µL	2 µL

6. Add 7 µL of the Fragmentation Master Mix to each tube.
7. Mix thoroughly by pipetting 8 to 10 times, spin and place on ice.
8. Place the tubes in a pre-warmed thermal cycler programmed to run Program 8 (cDNA Fragmentation, see Table 13):

   37°C – 30 min, 95°C – 2 min, hold at 4°C
9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

V. Labeling with the Encore Biotin Module

10. Continue immediately with the Biotin Labeling protocol.

F. Biotin Labeling

1. Make a master mix by combining FL3, FL4 and FL5 in a 0.5 mL capped tube, according to the volumes shown in Table 15.

Use Labeling Master Mix immediately after preparation

Mix by pipetting and spin down the master mix briefly at low speed. Place on ice. Use master mix immediately.

Table 15. Labeling Master Mix (volumes listed are for a single reaction)

LABELING BUFFER MIX (ORANGE: FL3)	LABELING REAGENT (ORANGE: FL4)	LABELING ENZYME MIX (ORANGE: FL5)
15 µL	1.5 µL	1.5 µL

2. Add 18 µL of the Labeling Master Mix to each cDNA Fragmentation reaction tube.
3. Mix thoroughly by pipetting 8 to 10 times, spin and place on ice.
4. Place the tubes in a pre-warmed thermal cycler programmed to run Program 9 (Labeling, see Table 13):

   37°C – 60 min, 70°C – 10 min, hold at 4°C
5. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
6. The labeled cDNA may be used immediately for array hybridization or stored at –20°C. For recommendations on array hybridization, refer to Appendix A.

VI. Technical Support

For help with any of our products, please contact NuGEN Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only). You may also send faxes to 888.296.6544 (toll-free) or email techserv@nugeninc.com.

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe@nugeninc.com.

In all other locations, contact your NuGEN distributor for technical support.

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A. Target Preparation for Array Hybridization

Note: Requires Affymetrix Hybridization Wash Stain (HWS) Kit for Gene 1.0 ST and Exon 1.0 ST Arrays

In general, cDNA targets amplified using the Applause WT-Amp Plus ST System and labeled using the Encore Biotin Module are prepared for analysis on GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays according to the Affymetrix GeneChip Whole Transcript (WT) Sense Labeling Assay User Manual (P/N 701880 Rev. 5) unless otherwise noted below.

To prepare target for a single array, use a 1.5 mL microcentrifuge tube and mix at room temperature the target cDNA and hybridization cocktail components as indicated in Table 16 below. Heat denature the hybridization cocktail at 99°C for 2 minutes (not 5 minutes as specified by Affymetrix), then follow the Affymetrix standard protocol (45°C in a heat block for 5 minutes then centrifuge at maximum speed for 1 minute just prior to loading). For the GeneChip Gene 1.0 ST Arrays (169 format), use a 90 µL hybridization volume. For the GeneChip Exon 1.0 ST Arrays (49 format), use a 200 µL hybridization volume.

We recommend a hybridization time of 18 hours +/– 2 hours. Hybridization times within this range yield comparable results. Choose the appropriate fluidics script for the chosen array type as noted in Table 16.

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Table 16. Hybridization Cocktail Assembly and Fluidics Protocol for GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays using the Affymetrix HWS Kit (Affymetrix P/N 900720)

COMPONENT	EXON 1.0 ST ARRAY (49 FORMAT)	GENE 1.0 ST ARRAY (169 FORMAT)	FINAL CONCENTRATION
Fragmented, biotin-labeled amplified cDNA	4 to 5 µg in 50 µL	2 to 2.5 µg in 25 µL	18.2 - 22.7 ng/µL
Control Oligonucleotide B2 (3 nM)	3.7 µL	1.8 µL	50 pM
20X Eukaryotic hybridization controls (bioB, bioC, bioD, cre)	11 µL	5.5 µL	1.5, 5, 25 and 100 pM, respectively
2x Hybridization Mix	110 µL	55 µL	1X
100% DMSO	22 µL	11 µL	10%
Water	23.3 µL	11.6 µL	
Final Volume	220 µL	110 µL	
FLUIDICS PROTOCOLS			
Fluidics Script	FS450_0001	FS450_0007	

B. Performing Quantitative PCR on Amplified cDNA

The amplified SPIA cDNA produced by the Applause WT-Amp Plus ST System has been successfully used as template for qPCR systems including TaqMan® and SYBR® Green.

For optimum results in qPCR applications, an aliquot of the amplified SPIA cDNA should be removed prior to Post-SPIA Modification where specified and used as template for qPCR as described here. Refer to pg. 15, step IV.G.9.

Note: RT-PCR master mixes containing the enzyme Uracil N-Glycosylase (UNG) are not compatible with the Applause WT-Amp Plus ST System.

We have successfully used the following master mixes for qPCR:

- TaqMan: ABsolute qPCR Mix plus ROX (ABgene, Cat. #AB-1136/B), Fast Universal PCR Master Mix 2X (Applied Biosystems, Cat. #4352042)

- SYBR: QuantiTect™ SYBR Green PCR Kit (QIAGEN, Cat. #204143), iQ SYBR Green Supermix (BioRad, Cat. #170-8880), FastStart SYBR Green Master (ROX) (Roche, Cat. #04 673 514 001)

Recommendations to Achieve Optimal Results

1. **Dilute the Amplified Product**
   The SPIA cDNA aliquot should be diluted 1:10 (minimum of 1:4) in 1 X TE or a buffer specified by the qPCR system manufacturer. A 2 µL aliquot of diluted SPIA cDNA is typically used per 25 µL qPCR reaction. Depending on the abundance of the transcripts you are measuring you may wish to dilute the cDNA further than 1:10 or use lower inputs of purified SPIA cDNA. It will be necessary to empirically determine the ideal input of amplified cDNA for use in a particular qPCR system.
2. **Primer Design**
   We recommend using primers and probes designed with amplicon sizes as small as possible. Primers may be designed at any position along a transcript since the Applause WT-Amp Plus ST System process covers the entire transcript.

C. Quality Control of Amplified cDNA Product

As a quality control test you may want to analyze the size distribution of the amplified cDNA product using an Agilent Bioanalyzer. Note that the shape of this distribution trace is highly dependent on the RNA source as well as input RNA integrity. We recommend using an RNA 6000 Nano LabChip (Agilent Cat. #5065-4476) and the Eukaryotic Total RNA Nano program (Nano assay in the Expert 2100 software) following the manufacturer's instructions. Depending on the availability of amplified product you may choose to load less than 100 ng of purified ST-cDNA product on the Bioanalyzer chip.

D. DNase Treatment of RNA

DNase Treatment During Purification: Using the QIAGEN RNase-Free DNase Set and the RNeasy Mini RNA Purification Kit

1. Homogenize the sample in RLT buffer including β-mercaptoethanol according to the type of sample as described in the RNeasy Mini Kit protocol.
2. Add 1X volume of 70% ethanol to the homogenized lysate, pipet up and down to mix sample well. Do not centrifuge.
3. Place an RNeasy mini column in a 2 mL collection tube.
4. Apply the sample (up to 700 µL), including any precipitate that may have formed, to the column.

5. Close the tube gently and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
6. For volumes greater than 700 µL, load aliquots onto the RNeasy column successively and centrifuge as before.
7. Add 350 µL Buffer RW1 into the RNeasy mini column to wash, and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
8. Add 10 µL DNase I to 70 µL Buffer RDD. Gently invert the tube to mix.

   Note: Other DNase I enzymes we can recommend to use in this step are the Shrimp DNase (recombinant) from USB Corp. (use 10 µL) , or the DNase I (RNase-free) from New England BioLabs (use 10 µL). See the Additional Reagent section of this user guide for ordering information.
9. Pipet the DNase I incubation mix (80 µL) directly onto the membrane inside the RNeasy mini column. Incubate on the bench top (~25°C) for 15 min.
10. Add 350 µL Buffer RW1 into the RNeasy mini column and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm) to wash. Discard the flow-through.
11. Transfer the RNeasy column to a fresh 2 mL collection tube. Add 500 µL Buffer RPE (with the added ethanol) to the RNeasy column.
12. Close the tube gently and centrifuge for 15 seconds at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
13. Add another 500 µL Buffer RPE to the RNeasy column.
14. Close the tube gently and centrifuge for 2 minutes at ≥8000 X g (≥10,000 rpm). Discard the flow-through.
15. Transfer the RNeasy column to a new 1.5 mL collection tube.
16. Pipet 30–50 µL RNase-free water directly onto the RNeasy membrane.
17. Close the tube gently and centrifuge for 1 minute at ≥8000 X g (≥10,000 rpm) to elute.
18. If yields of greater than 30 µg are expected, repeat elution step and collect in the same collection tube.

DNase Treatment of RNA Post-Purification: Using RNase-free DNase and Either the RNA Clean and Concentrator™-5 Columns or the RNeasy MinElute Columns

Note: If you are unable to quantify your RNA because the sample is contaminated with DNA, we recommend DNase treatment followed by purification.

1. On ice, mix together 2.5 µL 10X DNase I Reaction buffer (Roche Cat. #04716728001 or USB PN 78316) with 1 µL rDNase (10 Units Roche Cat. #04716728001 or 2 Units USB PN 78311).

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2. Add RNA sample (up to 500 ng) and add Nuclease-free Water (D1, green cap) to bring the final volume to 25 µL.
3. Incubate at 25°C for 15 minutes followed by 37°C for 15 minutes and return to ice.
4. After the DNase treatment, the sample must be purified. We recommend either of the two purification procedures below:

Purification with RNA Clean and Concentrator-5
(Zymo Research, Cat. #R1015)

a. Add 4 volumes (100 µL) of RNA binding buffer to the sample.
b. Obtain one RNA Clean and Concentrator-5 column and apply sample to column.
c. Spin column for 30 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
d. Add 200 µL wash buffer (with ethanol added as per vendor's specifications).
e. After closing the column, spin for 30 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
f. Add 200 µL fresh 80% ethanol, close cap, spin for 30 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
g. Place the RNA Clean and Concentrator-5 column in a fresh 1.5 mL collection tube.
h. Add 10 µL Nuclease-free Water (green: D1) directly to the center of the filter in the tube and close the cap. **Do not use cold water!**
i. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

Use nuclease-free water at room temperature to elute sample.

Purification with QIAGEN RNeasy MinElute Cleanup Columns
(QIAGEN, Cat. #74204)

a. Add 80 µL ice-cold Nuclease-free Water (D1, green cap) to the sample on ice.
b. Add 350 µL Buffer RLT and mix by pipetting.
c. Add 250 µL 96–100% ethanol and mix thoroughly by pipetting.
d. Place an RNeasy MinElute Spin Column into a 2 mL collection tube (one column per sample) and apply the 700 µL sample to the column.
e. After closing the column, spin for 15 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through.
f. Place the RNeasy MinElute Spin Column into a fresh 2 mL collection tube. Add 500 µL Buffer RPE to the column and close the tube. Spin for 15 seconds at ≥8000 X g (≥10,000 rpm) and discard the flow-through, keeping the same collection tube.
g. Add 500 µL 80% ethanol to the RNeasy MinElute Spin Column and close the tube.

Best results can be obtained by using fresh 80% ethanol in the wash step. Lower percent ethanol mixes will reduce recovery.

Note: Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.

h. Spin for 2 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.

i. Place the RNeasy MinElute Spin Column in a fresh 2 mL collection tube and place in the microcentrifuge with the cap open. Spin for 5 minutes at ≥8000 X g (≥10,000 rpm) and discard the flow-through.

j. Place the RNeasy MinElute Spin Column in a fresh 1.5 mL collection tube.

Use nuclease-free water at room temperature to elute sample.

k. Add 14 µL Nuclease-free Water (D1, green cap) directly to the center of the filter in the tube and close the cap. **Do not use cold water!**

l. Spin for 1 minute at ≥8000 X g (≥10,000 rpm) to collect the purified RNA.

E. Preventing Non-specific Amplification

Due to the high sensitivity inherent in our amplification systems, we have developed a set of recommendations designed to minimize the potential for the generation of non-specific amplification products through the carry-over of previously amplified SPIA cDNA. We strongly recommend implementing these procedures, especially for the high-throughput and low-RNA input environments typical in today's gene expression laboratories.

We have two general recommendations. First, designate separate workspaces for "pre-amplification" and "post-amplification" steps and materials. This provides the best work environment for processing RNA using our highly sensitive amplification protocols. Our second recommendation is to implement routine clean-up protocols for workspaces as standard operating procedure. This will prevent non-specific amplification products from spreading through the laboratory. Details regarding establishing and maintaining a suitable work environment are listed below:

1. Designate a pre-amplification workspace separate from the post-amplification workspace or general lab areas:

   a. Pre-amplification includes all steps and materials related to RNA sample handling and dilution, NuGEN's first strand reaction, second strand reaction, second strand cleanup and SPIA amplification reaction setup. After SPIA incubation the reactions are immediately removed from the pre-amplification workspace and opened only in the post-amplification area.

   b. Post-amplification includes all steps and materials related to the handling of the final amplified cDNA product including bead removal, final purification, post-SPIA modification, array hybridization and any other analytical work.

   c. Ideally the pre-amplification workspace will be in a separate room. If this is not possible, ensure the pre-amplification area is sufficiently isolated from post-amplification work.

d. PCR Workstation enclosures with UV illumination for use as pre-amplification workspaces can be an option in situations where conditions preclude physical separation of pre- and post-amplification activities.

2. Establish and maintain a clean work environment:

   a. Initially clean the entire lab thoroughly with DNA-OFF. Follow this treatment with a thorough rinse with water to ensure no residual cleaning agents are left behind.

   b. In the pre-amplification area, remove all small equipment, and then clean every surface that may have been exposed to amplified SPIA cDNA (surfaces, drawer handles, key pads, etc.). Before reintroducing any equipment, clean every piece of equipment thoroughly.

      - Clean thermal cycler blocks by heating to 99°C for 15 minutes, then wipe down exposed surfaces and keypad with cleaning solution.
      - Clean magnets by immersion in cleaning solution or use a cotton swab.

   c. Carry out a thorough external and internal cleaning of all pipettes with DNA-OFF. Carefully follow the manufacturer's instructions for this process to avoid damaging the pipettes. It is a good idea to keep a clean set of pipettes as a backup.

   d. Always wear gloves and don fresh gloves upon entry into this controlled area. Frequently change gloves while working in the pre-amplification area, especially prior to handling stock reagents, reactions and RNA samples.

   e. Stock this area with clean (preferably new) equipment (pipettes, racks, consumables) that has not been exposed to post-amplification workspace.

   f. Make it a policy to carry out regular cleaning of all workspaces.

   g. Capture waste generated in both pre- and post-amplification areas (tips, columns, wash solutions from beads and columns, tubes, everything) in sealable plastic bags and dispose of promptly after each experiment to avoid waste spillage.

   h. Do not open amplified product reaction vessels in the pre-amplification workspace.

3. Avoid running negative controls (i.e., no RNA input reactions). Instead use low-template controls (inputs of 50 pg to 100 pg) in order to detect and monitor any non-specific amplification issues. The clearest indication that non-specific amplification is taking place is the appearance of higher than expected yields or irregular Bioanalyzer traces in a low template control (LTC) reaction.

   a. Typical amplification performance:

      i. LTC yields for Applause WT-Amp Plus ST System amplifications should be significantly lower than yields for RNA inputs within the recommended input range of 50 ng to 200 ng.

ii. The Bioanalyzer trace of the LTC amplification product is consistent with that seen with higher input.

b. Atypical amplification performance:

i. LTC yields may be similar to those obtained using higher inputs of total RNA.

ii. The Bioanalyzer traces of amplification products may look significantly different than the typical Applause WT-Amp Plus ST System reaction traces. The LTC reaction is designed to be an especially sensitive indicator of atypical amplification performance.

iii. Sensitivity on arrays may be lower than expected.

iv. Contact NuGEN Technical Services when atypical performance is suspected.

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F. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the Applause WT-Amp Plus ST System?
The Applause WT-Amp Plus ST System provides all necessary buffers, primers and enzymes for first strand cDNA synthesis, second strand cDNA synthesis and amplification, and all necessary buffers and enzymes for converting amplified cDNA into sense target cDNA (ST-cDNA). For your convenience, nuclease-free water has also been included.

Q2. What additional consumables does the user need?
For the ST-cDNA purification step, the QIAGEN MinElute Reaction Cleanup Kit, Catalog #28204, is required. The user guide also lists recommendations for specific consumables, including nuclease-free pipette tips, nuclease-free microcentrifuge tubes, 0.2 mL PCR tubes and plates, RNaseZap and DNA-OFF.

Q3. What is the minimum input required for amplification? Is there a maximum input?
The Applause WT-Amp Plus ST System can be used with high-quality, purified total RNA in the range from 50 to 200 ng. Input amounts outside this range may produce unsatisfactory and variable results.

Q4. Can I amplify degraded RNA with the Applause WT-Amp Plus ST System?
The Applause WT-Amp Plus ST System is not designed for use with degraded RNA. Using compromised samples will result in unsatisfactory and variable results.

Q5. How much cDNA can I expect from a single reaction?
You should expect a minimum yield of 4 µg for the Applause WT-Amp Plus ST System when used as directed.

Q6. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, thermal cycler and a UV-Vis spectrophotometer. An Agilent Bioanalyzer or similar instrument may be used for quality control.

Q7. Does the Applause WT-Amp Plus ST System provide any labeling reagents?
No. The Applause WT-Amp Plus ST System is used to generate ST-cDNA from total RNA for use in gene expression experiments. The resulting ST-cDNA may be processed further using the Encore Biotin Module for labeling and analysis on Affymetrix GeneChip Gene 1.0 ST and Exon 1.0 ST Arrays.

Q8. Where can I safely stop in the protocol?
You may stop immediately following the SPIA Amplification protocol, or after Post-SPIA Modification II protocol prior to final cleanup at the points specifically noted in the protocol. Store reaction products at –20°C.

Q9. What are the recommended storage conditions for the Applause WT-Amp Plus ST System components?
All components of the system may be stored at –20°C. Ensure the vials are well sealed and do not exceed 6 freeze/thaw cycles.

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Q10. What are the recommended storage conditions for the amplified ST-cDNA?
The amplified ST-cDNA may be stored at –20°C.

Q11. Can DNA be used as input for the Applause WT-Amp Plus ST System?
No. The Applause WT-Amp Plus ST System is designed to amplify mRNA, not DNA.

Q12. Has NuGEN performed reproducibility studies on the Applause WT-Amp Plus ST System?
Yes. Sample-to-sample, lot-to-lot and operator-to-operator reproducibility studies are routinely conducted according to NuGEN's internal Quality Control metrics.

Q13. Can contaminating genomic DNA interfere with the performance of the Applause WT-Amp Plus ST System?
Yes. In high quantities, genomic DNA will interfere with amplification.

Q14. Does NuGEN recommend DNase treatment of purified total RNA samples?
Yes. For DNase treatment of RNA samples, refer to Appendix D of the user guide.

Q15. Can I use the Applause WT-Amp Plus ST System on bacterial RNA samples?
The amplification process theoretically will work with many bacterial species; however, the kit has not been optimized for this purpose and NuGEN cannot guarantee success with such samples.

Q16. Can I use the Applause WT-Amp Plus ST System for archiving cDNA?
Yes. Amplified cDNA may be safely stored at –20°C for six months or longer.

Q17. How do I quantitate the amplified cDNA product?
You may use a standard UV-Vis spectrophotometer or a NanoDrop. Be sure to use the single-stranded cDNA conversion factor of 1 A260 unit = 33 ng/µL in calculating the amplified cDNA concentration as this is the convention we used in establishing yield guidelines in this user guide.

Q18. Why do I need to use the single-stranded cDNA conversion factor when converting my A260 reading to cDNA concentration?
The amplified cDNA product of the Applause WT-Amp Plus ST Systems consists of both sense and antisense cDNA strands. While there may be some double-stranded character to this mixture, we have developed and optimized the kit using the single-stranded cDNA conversion factor (1 A260 unit = 33 ng/µL of cDNA). Expected cDNA yield from amplification and input into labeling protocols cited in the product materials all have been generated using this convention.

Q19. How many rounds of amplification are performed in the Applause WT-Amp Plus ST System?
This System performs a single round of amplification. It is not designed to support multiple rounds of amplification.

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Q20. Do I need to order specific primers for the amplification?
No. The DNA/RNA primers provided in the Applause WT-Amp Plus ST Systems are universal. No gene-specific primers are required.

Q21. Do I have to use the supplied DNA/RNA primers?
Yes. The Applause WT-Amp Plus ST System will not work properly with other primers.

Q22. Do you recommend purification of the amplified ST-cDNA prior to qPCR analysis?
No. The recommendations given in Appendix B of the user guide describe the use of diluted, unpurified SPIA cDNA as the optimal template for qPCR reactions.

Q23. Where in my target sequence can I design qPCR primers?
The Applause WT-Amp Plus ST System amplifies the entire transcript so primers can be designed at any location within the mRNA. In order to avoid interference from possible genomic DNA contamination, we recommend treating RNA with DNase and designing amplicons to span an intron.

Q24. How many qPCR reactions will I get from one Applause WT-Amp Plus ST amplification?
The number of qPCR reactions depends on the abundance level of the genes being interrogated and the size of the SPIA cDNA aliquot set aside for this purpose. For medium- to high-copy number genes, the cDNA may be substantially diluted. For very-low-copy number genes you may need to use more cDNA per reaction.

Q25. What is the recommended minimum batch size?
We recommend a minimum batch size of eight reactions. Smaller batches may result in poor performance due to the challenge of accurately pipetting small volumes.

Q26. Can I amplify RNA that has been isolated with the aid of a carrier?
Many common carriers will interfere with the amplification process. Glycogen inhibits reverse transcriptase and yeast tRNA will produce cDNA in the first strand synthesis and interfere with the analysis. We typically don't recommend using a carrier, but if it is unavoidable then please contact the NuGEN Technical Support Team for information on compatible carriers.

VII. Appendix

G. Update History

This document, the Applause WT-Amp Plus ST System user guide (M01199 v2.1) is an update to a previous version to address the following topics:

Description	Section	Page(s)
Updated SPIA technology description.	I.B.	1
Updated contact information for NuGEN Technical Support.	VI.	23

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